

Exhibit 2



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Topical Antimicrobial Toxicity

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Three topical antibiotics and four antiseptics (1% povidone-iodine, 0.25% acetic acid, 3% hydrogen peroxide, and 0.5% sodium hypochlorite) were directly applied to cultured human fibroblasts to quantitatively assess their cytotoxicity. The four antiseptics were found to be cytotoxic; all of the cytotoxic agents except hydrogen peroxide were subsequently found to adversely affect wound healing in an animal model. Comparison of bactericidal and cytotoxic effects of serial dilutions of these four topical agents indicated the cellular toxicity of hydrogen peroxide and acetic acid exceeded their bactericidal potency. Bactericidal noncytotoxic dilutions of povidone-iodine and sodium hypochlorite were identified. These experiments provide evidence that 1% povidone-iodine, 3% hydrogen peroxide, 0.5% sodium hypochlorite, and 0.25% acetic acid are unsuitable for use in wound care. This sequence of experiments could be used to identify bactericidal, noncytotoxic agents prior to their clinical use.

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Many more surgeons know how to cause suppuration than to heal a wound.

HENRY DE MONPEVILLE (1260-1320)

Topical antimicrobials are commonly used in all varieties of wounds as adjuncts to surgery and as chronic treatments. The effects of these substances on living tissue and the healing processes are disputed; claims of toxicity and lack of toxicity rest on sometimes contradictory clinical and laboratory reports.

We have used a series of in vitro and in vivo experiments to quantitate cytotoxicity, bacterial toxicity, and the effects on wound tensile strength and epithelialization of commonly used topical agents.

METHODS

Topical Agents

Three antibiotics (bacitracin, 50 units/mL; 1% neomycin sulfate; and 2% kanamycin sulfate) and four antiseptic agents (1% povi-

done-iodine,

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done-iodine, 0.25% acetic acid, 0.5% sodium hypochlorite, and 3% hydrogen peroxide) were studied.

Sequence of Experiments

Cultured human fibroblasts have been used to quantitate antibiotic toxicities.¹ We used cultured fibroblasts as a quantitative assay of cytotoxicity of topical agents. This assay is comparable with in vitro bactericidal assessments of topical agents,² an aspect of topical antimicrobials previously well studied.³ Agents found to be toxic to fibroblasts were then applied to an in vivo wound model to see if the observed in vitro toxicities were reflected in animal studies. Finally, fibroblast and bacterial toxicities were directly compared in parallel fibroblast and bacterial assays.

Fibroblast Toxicity Assay

Human fibroblasts were obtained from newborn human foreskin. Confluent cultures raised in RPMI-1640 (Gibco, Grand Island, NY) were trypsinized using 0.5% trypsin (Gibco, Grand Island, NY), washed in balanced salt solution, and divided into two equal aliquots, each containing approximately 2.5×10^6 cells. Each portion was centrifuged at 100 g for ten minutes. The resulting cell button was suspended in either saline as a control or in a topical agent. After 15 minutes, the cells were again centrifuged, washed, suspended in RPMI-1640, and incubated in 25-ccm culture flasks (Corning, Corning, NY) for 24 hours. Cell viability was assessed at the initiation of incubation by staining a small sample of cells with a vital dye (trypan blue) and expressed as a percentage of viable cells seen among total cells counted. After 24 hours of incubation, living cells in cultures exposed to topical agents were counted and expressed as a percentage of living cells found in the cultures of cells exposed to saline. If toxicity was evident after exposure to an agent, the agent was serially diluted until no toxicity was observed.

Wound Studies

Adult, female Sprague-Dawley rats (Dominion Laboratory, Dublin, Va) were divided into six groups of 20 animals. The animals were anesthetized with 30 mg/kg of sodium pentobarbital. Standard 4-cm wounds were made transversely across the midline of the back 1 cm cephal to the base of the neck. The wounds transected all tissue down to the fascia of the back muscles. The wounds were left open and the wound areas recorded by tracing them on transparency.

Five of the groups were irrigated three times a day with either saline, 1% povidone-iodine, 0.25% acetic acid, 0.5% sodium hypochlorite, or 3% hydrogen peroxide. Each irrigation consisted of

Table 1.—Survival of Cultured Human Fibroblasts 24 Hours After Exposure to Topical Agents

Agent	NP	% Fibroblast Survival at 24 hr (Mean \pm SEM)
Bacitracin, 0.5% unlabeled	3	113 \pm 8
1% neomycin sulfate	3	104 \pm 20
2% kanamycin sulfate	3	92 \pm 10
1% povidone-iodine	3	0
0.25% acetic acid	3	0
0.05% sodium hypochlorite	3	0
3% hydrogen peroxide	3	0

*N indicates number of cultures tested.

washing the wound with 15 mL of the test solution so that the wound was visibly soaked. One group received no irrigation.

At intervals of 4, 8, 12, and 16 days after wounding, all the animals were anesthetized and the unepithelialized portions of their wounds were traced on transparency. The actual area was calculated by computer analysis of the transparency tracings. The percentage of unepithelialized wound was calculated at each time interval by comparing the area of the healing wound with the original area of the wound.

At each time interval, five rats from each group were randomly killed and the central 2 cm of wound carefully excised. Wound thickness was measured at the center of the wound with a micrometer. Breaking strength was determined on a simple apparatus originally described by Crawford et al.⁶ and tensile strength was calculated by dividing the breaking strength by the cross-sectional area of the wound.

Bacterial Toxicity Assay

Paired aliquots (each containing approximately 2.0×10^6 organisms) of *Staphylococcus epidermidis* cultured in Todd-Hewitt broth (Difco, Detroit, Mich) were suspended in either saline or a topical agent for 15 minutes, centrifuged at 200 g for two minutes, washed, and suspended in 5 μ L of saline. These suspensions were serially diluted, plated on agar culture medium, and incubated for 24 hours. Results were expressed as a percentage of colonies found at 24 hours in the cultures of bacteria exposed to a topical agent compared with the colonies in the cultures of bacteria exposed to saline. Different concentrations of each test solution were used to determine maximal and minimal bactericidal concentrations.

Data Analysis

The significance of differences noted between the agents and concentrations tested in the above experiments was estimated by Student's *t* test.

RESULTS

Fibroblast Toxicity

At full strength, none of the antibiotics were toxic to fibroblasts; all of the antiseptics were 100% cytotoxic (Table 1).

Serial dilutions of the four antiseptics were assayed for cytotoxicity (Figure). Significant decreases ($P < .01$) of fibroblast survival in culture persisted with concentrations of 0.025% sodium hypochlorite (0% fibroblast survival), 0.05% povidone-iodine (50% \pm 25% fibroblast survival), 0.25% acetic acid (74% \pm 7% fibroblast survival), and 0.03% hydrogen peroxide (41% \pm 7% fibroblast survival). No decreases of

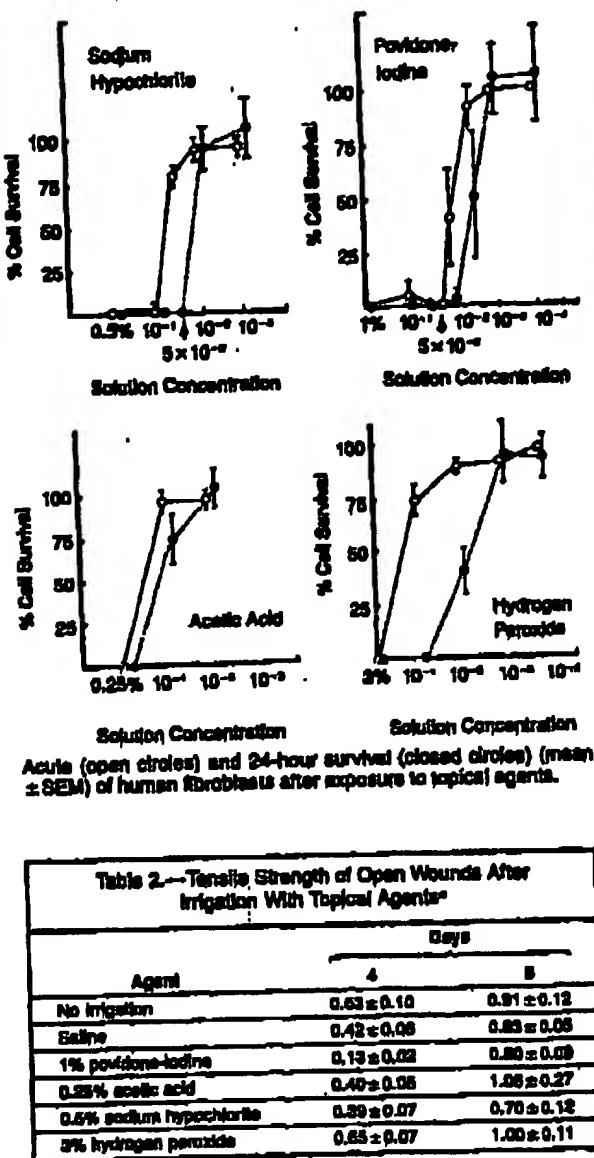


Table 2.—Tensile Strength of Open Wounds After Irrigation With Topical Agents*

Agent	Days	
	4	8
No irrigation	0.63 \pm 0.10	0.91 \pm 0.12
Saline	0.42 \pm 0.08	0.33 \pm 0.05
1% povidone-iodine	0.13 \pm 0.02	0.20 \pm 0.09
0.25% acetic acid	0.40 \pm 0.05	1.06 \pm 0.27
0.05% sodium hypochlorite	0.39 \pm 0.07	0.70 \pm 0.12
3% hydrogen peroxide	0.65 \pm 0.07	1.00 \pm 0.11

*Mean \pm SEM, grams per square millimeter.

fibroblast survival in culture were found with 0.005% sodium hypochlorite, 0.001% povidone-iodine, 0.025% acetic acid, and 0.003% hydrogen peroxide. Vital staining at the initiation of incubation did not correctly predict cell survival in culture after serial dilution of cytotoxic agents.

Wound Studies

At four days, wounds irrigated with 1% povidone-iodine were significantly ($P < .01$) weaker than wounds irrigated with saline, other topical agents, or unirrigated wounds (Table 2). Tensile strength of wounds irrigated with povidone-iodine was only 21% that of control wounds. There

Table 3.—Percentage of Unepithelialized Wounds After Irrigation With Topical Agents*

Agent	Day			
	4	8	12	16
No irrigation	50.0±0.0	10.5±1.8	5.0±1.3	1.4±0.3
Saline	55.0±4.2	10.0±2.2	4.7±1.5	0.6±0.5
1% povidone-iodine	55.7±3.9†	30.4±4.9†	10.1±1.4	2.4±1.5
0.5% acetic acid	75.0±2.7†	30.7±3.0†	4.5±1.8	0.4±0.3
0.05% sodium hypochlorite	69.0±7.4	37.0±5.0†	11.0±2.3	0.0±0.0†
3% hydrogen peroxide	60.0±6.8	15.0±1.8	5.2±1.1	0

*Mean ± SEM.

†Indicates a significant ($P < .05$) difference from saline and control values.

Table 4.—Comparative Bacterial and Fibroblast Toxicities of Topical Agents*

Agent and Concentration	% Fibroblast-Survived at 24 hr†	% Bacterial Survival at 24 hr†	P Value
Povidone-iodine, %			
0.01	0 (0)
0.001	105±6.6 (3)	0 (0)	<.001
0.0001	100±12 (3)	103±5	...
Sodium hypochlorite, %			
0.00	0 (4)
0.005	87±6 (5)	0 (4)	<.001
0.0005	107±12 (5)	71±5 (5)	<.01
0.00005	...	118±8	...
Hydrogen peroxide, %			
3.0	0 (3)	0 (4)	...
0.3	0 (3)	103±5 (3)	<.001
0.03	41±7 (3)	105±8 (3)	<.01
0.003	98±2 (3)	95±4 (3)	...
Acetic acid, %			
0.5%	0 (4)	78±3 (4)	<.001
0.05%	74±7 (5)	97±2 (4)	<.05
0.0005%	105±5 (3)

*Numbers in parentheses represent the numbers of experiments performed.

†Mean ± SEM.

were no significant differences at 8, 12, or 16 days.

Wound epithelialization was significantly retarded at four days by povidone-iodine and acetic acid; at eight days by povidone-iodine, acetic acid, and sodium hypochlorite; and at 16 days by sodium hypochlorite (Table 3).

Bacterial Toxicity

One hundred percent bactericidal dilutions and nonbactericidal dilutions were identified for the four cytotoxic topical agents. When bactericidal activities of serial dilutions of topical agents were compared with fibroblast cytotoxicities at the same strengths, 1% povidone-iodine and 0.5% sodium hypochlorite proved to have bactericidal, nontoxic concentrations (0.001% and 0.005%, respectively); while with hydrogen peroxide and acetic acid, fibroblast toxicity exceeded bacterial toxicity (Table 4).

COMMENT

Previous investigations of cytotoxicity of topical agents have used qualitative or indirect methods. Brannmark described microcirculatory damage following applications of

antiseptic compounds to living animal tissue¹; Edlich et al² used susceptibility of wounds to bacterial infection as an indicator of local injury after topical agent exposure.² Quantitative studies of the effects of topical agents on wound healing have included epithelialization rates (in days) following cotton-tipped applicator administration of topical agents³; breaking strengths of either "closed"⁴ or "open"⁵ wounds after single applications of povidone-iodine; and quantitated epithelialization of open wounds packed with povidone-iodine-soaked dressings.⁶ None of these quantitative studies have documented compromise or enhancement of wound healing following the application of topical agents. Clinical reports have presented conflicting data. In the case of povidone-iodine, for example, there are recent reports of increased,⁷ decreased,⁸ and unchanged⁹ rates of wound complications following its use.

Our fibroblast toxicity model provided quantitative data indicating marked cytotoxicity of four commonly used topical antimicrobials as follows: povidone-iodine, acetic acid, hydrogen peroxide, and sodium hypochlorite. Toxicity of antibiotic agents was not demonstrated. Of the four

cytotoxic agents, three of them could be shown to have deleterious effects on the healing of open skin wounds in rats. The one cytotoxic agent that did not retard healing in the animal model, hydrogen peroxide, also had minimal bactericidal potency (Table 4). These findings argue against the use of these agents as useful adjuncts in wound care. Our finding of bactericidal, noncytotoxic dilutions of two of these agents, povidone-iodine and sodium hypochlorite (Table 4), indicate an approach to defining safe strengths of topical agents that might be further investigated in animal studies or clinically.

In 1968, King and Price¹¹ observed that "... the overall

history of skin antisepsics may be viewed as a repetitious story of antibacterial agents enthusiastically introduced, uncritically and widely adopted, subjected in time to more critical evaluation, and eventually discarded by a disillusioned or discredited profession." Critical evaluation in the form of cytotoxicity and bactericidal studies, as well as in vivo wound studies, have provided in our experiments quantitative evidence of the unsuitability of four commonly used topical agents for wound care. The same sequence of experiments could be applied to the identification of safe dilutions of topical agents or new noncytotoxic topical agents prior to their application in clinical practice.

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Discussion

J. PATRICK O'LEARY, MD, Nashville, Tenn: First, I would like to compliment Dr. Linawaeaver for a very nice project and also the use of apparatus which defies technology. The second point I would like to make is that we are probably very fortunate that Dr. Linawaeaver and his particular studies were not available to Joseph Lister when he did his original studies with carbolic acid.

I have a couple of comments to make and then a couple of questions. We recently published our experience in the Newark VAMC. Dr. Linawaeaver, in his discussion of that article, pointed out that there is a toxic effect of povidone-iodine when it is irrigated through wounds. This study has proved his contention.

Any antimicrobial has its effect by its action either on the metabolic pathway in the living cell or on the surface membrane of the bacteria. Therefore, all antibiotics have an antimicrobial effect. It would be anticipated that, if appropriate conditions are applied, any such agent could be toxic to a healing wound. I believe this study clearly points out that there is an aperture where the desired effect can be obtained without producing cellular damage. In the healing wound, we are trying to produce that situation where the tensile strength will be adequate in holding the wound together and, at the same time, produce a hostile milieu for bacterial growth. I would not interpret your study as showing that topical antibiotics are bad, but I would interpret your study as showing that certain concentrations of antimicrobials are appropriate.

My questions are as follows: Do you look at anything other than fibroblast survival in wound healing? Do you have data to support the concept that there is a direct relationship between fibroblast activity and wound healing? Did you measure the hydronium ion content of these wounds? Did you calculate tensile strength as a measure of cross-sectional area? I think it is a very lovely study, and I compliment you for it.

DR. LINAWAEVER: Raising the name of Lister is, I think, an important point. Use of topical substances going back to Lister's carbolic acid has to be looked at within the context that, initially,

part of what Lister and the various proponents of his thinking have been proposing by using topical agents is simple skin disinfection. Skin is pretty tough and can tolerate a lot of things without much obvious damage. However, using these substances in open wounds is actually another issue, one we are actually trying to address here. Lister's work preceding to Carrel's work with Dakin's solution in World War I and experiences in the early 1900s, during the Spanish Civil War, were done within the context of not being able to do anatomic debridement, because of lack of intravenous fluid technology, anesthesia support, and other various limitations of the battlefield. Surgeons were forced simply to leave large amounts of necrotic tissue on and they would begin pouring things on the tissue and see variable effects. I think a whole branch of the Listerian descendant thinking has been based on this extremely chaotic although somewhat heroic attempt to deal with disastrous wounds under disastrous situations. It simply does not hold up when compared with a true anatomic debridement of a widely affected area. So, if I had been able to discuss this with Lister at the time, I would have tried to make a distinction between skin disinfection and treatment of wounds.

To specifically answer the questions as I recorded them: Yes, the point of concentration is the one we are trying to make by comparing the antibacterial and the cytotoxic activity. We were able to find that there were two potentially safe dilutions, one of povidone-iodine and one of good old Dakin's solution, that appear to kill bacteria while not injuring cells. In part of our summary, we specified this approach for identifying safe concentrations. So, yes, there do appear to be concentrations of these agents that are more damaging than others.

We did not do biochemical assays. We simply looked at *in vitro* fibroblast damage and then looked at our wound studies.

Tensile strength is the breaking strength of the wound divided by the cross-sectional area of the wound, and our data derive from that calculation.